

Panorama® Antibody Array Technology

General Information

Why study protein expression?

- Advances in technology over the past decade have enabled the acquisition of a wealth of information contributing to understanding the genome. These techniques primarily involved the study of mRNA.
- mRNA undergoes a number of alternative processing steps prior to and following translation. There is often poor correlation between mRNA and protein expression,¹ and this has driven development of platform technologies that enable measurement of biologically-relevant changes in expression at the protein level.
- Antibody arrays enable researchers to directly compare the expression levels of hundreds of target proteins from a variety of cell or tissue extracts in half a day.

Panorama Antibody Array Technology

- Panorama Antibody Arrays are complete systems for multiplex profiling of protein expression in biological samples.
- The arrays consist of polyclonal and monoclonal antibodies spotted in duplicate on nitrocellulose-coated glass slides.
- Antibodies are selected for characteristics of high specificity and recognize human, mouse and rat proteins.
- The nitrocellulose-coated slides are treated with a proprietary blocker to ensure optimal signal-to-noise ratio.
- Antibody content is specific to the particular theme of a Panorama Array product. Each theme covers specific biological pathways, including: gene regulation, cell signaling, MAPK & PKC pathways, or p53 pathways.
- Panorama Array products are supplied as complete kits* comprising:
 - 2 array slides
 - Cell and tissue extraction/labeling buffers
 - Protease and phosphatase inhibitor cocktails
 - Post-reaction clean-up columns
 - All plasticware (cell culture vessels, 2 mL polypropylene tubes) necessary to conduct a 2-array assay
 - CD with list and .GAL file for data analysis

* Kits do not contain a reactive fluorophore reagent for labeling. Cyanine (Cy™) dyes from GE Healthcare are recommended in the technical bulletin.

Applications

- Sample response to external treatments (e.g., starvation, addition of growth factors, serum, drugs/biomolecules).
- Comparison of different cell or tissue samples (clinical specimens, tissue, heart, brain, lung etc.).
- Gene silencing (RNAi) validation, off-target effects.

1. Gygi, S.P., et al. (1999) *Mol. Cell. Biol.*, 19, 1720-1730.

Protein Expression in F9 Mouse Stem Cells Induced by Retinoic Acid

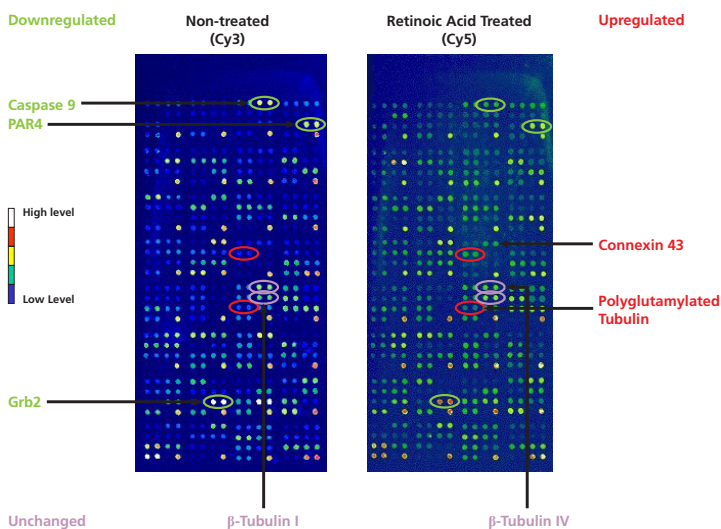


Figure 1. Expression of proteins in F9 mouse stem cells induced to differentiate by retinoic acid utilizing the Panorama Antibody Array-Cell Signaling. The total cell extract of F9 cells non-treated or treated for four days with retinoic acid were prepared and labeled with Cy3 and Cy5, respectively. Equal amounts of Cy3 or Cy5 labeled protein were incubated on the slide. The experiment was repeated at least three times. Proteins marked in red were up-regulated while those in green were down-regulated.

Differential Expression Between Different Tissue Types

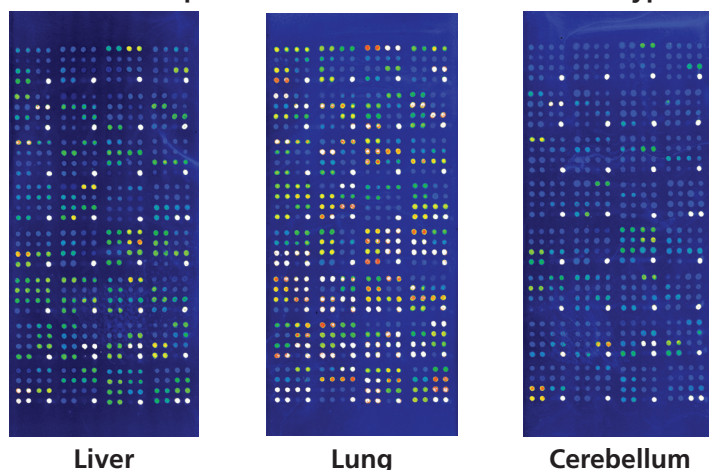


Figure 2. Differential protein expression in mouse tissues utilizing the Panorama Antibody Array-Cell Signaling. Equal concentration of mouse tissue extract (5 µg/mL) from liver, lung, and cerebellum were incubated on different slides.

Frequently Asked Questions

Do antibody arrays provide a quantitative analysis of protein expression?

- Antibody arrays are specific for a qualitative measurement of protein expression for tissue or cell extracts. By determining relative changes in protein expression, it is possible to compare a "reference" sample to a "test" sample and establish a ratio rendering it qualitative.
- It is recommended to use traditional protein quantitation techniques (Western blots, immunoblots, mass spectrometry) to validate data obtained from antibody arrays.

Can the antibody arrays be reused?

- Sigma does not recommend stripping the arrays and re-probing, as this may compromise the integrity of the antibodies.

Antibodies

Where can I find a list of the antibodies on an array?

- The complete list of antibodies and reactivity information (mouse, human, or rat; polyclonal or monoclonal) may be found on the product-specific landing pages at sigma-aldrich.com/arrays.

Are the antibodies available separately?

- **Yes.** All antibodies used on the arrays are available separately from Sigma-Aldrich. For more information visit: sigma-aldrich.com/arrays.
- All antibodies featured on Panorama® Antibody arrays are also validated for downstream protocols such as Western blots and immunoprecipitation.

What type of validation screening is performed to ensure recognition of the protein by the antibody?

- Each lot of antibody arrays is tested for recognition of Cy3 or Cy5 labeled proteins extracted from cell lines.

Sample Types

What type of samples can be used with the array?

- Antibody arrays are designed for studying protein expression in cell or tissue extracts and blood plasma or serum samples.
- Wherever possible, use of freshly prepared samples is recommended. It has been reported that assays have also been successfully performed using frozen archive samples.
- The antibodies spotted recognize primarily human, mouse, and rat proteins.

How can I determine protein concentration?

- As indicated in the technical bulletin, it is recommended to use the Bradford assay to determine protein concentration prior to applying samples to the antibody array.

Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248-254, 1976.

I have genomic DNA contamination.

- Add 0.6 units/mL of Benzonase® (supplied with kit).
- If DNA contamination still exists, Benzonase concentration may be increased 10x without deleterious effects on the protein sample.

Labeling Samples

What residues does the Cy label conjugate with on my protein?

- Cy3/Cy5 labeling is via lysines on the protein.

How much labeled protein should be applied to an array?

- The recommended range for protein concentration is 2-10 µg per mL of incubation medium in a 4 to 5 mL total volume. Therefore, the minimum amount recommended is 8 -10 µg of labeled protein. For the XPRESS Profiler725 we recommend using 10-30 µg/mL.

How much protein is required for efficient labeling?

- The manufacturer's published protocol for Cy3/Cy5 labeling describes using 1 mg of total protein.
- For low abundance proteins, and if quantity of sample permits, it is highly recommended to label concentrated protein samples (5-10 mg/mL) and then dilute appropriately for the assay.
- Reaction volumes for the labeling procedure may be scaled down by maintaining the molar ratios of the reagents as described in the original protocol. It is advised, however, to conduct initial array experiments according to published protocols to ensure a valid result is obtained and allow a baseline to be determined.

What is the recommended dye to protein (D/P) ratio?

- In order to obtain the expected performance as described by the technical bulletin, it is recommended to use a D/P ratio >2.

I cannot achieve a D/P ratio >2.

- Quantity of starting sample used may be too low, increase the labeling time in half-hour increments (i.e., 1 hr; 1.5 hrs) to achieve optimal D/P ratio. A total incubation time of two hours may be required in some circumstances.
- Make sure the lysate sample is not viscous before incubating the dye with the lysate. Viscosity is the result of DNA that is not fully degraded. Add more Benzonase in 0.6 units/mL until the lysate is no longer viscous.
- Cy3 and Cy5 dyes are sensitive to light. Ensure reaction vessels are covered in aluminum foil or conduct the reaction in a darkroom.



Scanning Slides

What type of instrument/software is required to perform the assay?

- Antibody arrays can be scanned with most commercially available 'overhead' lightsource DNA array scanners that accommodate standard microscope slides.
- Scanners with bottom-lit light sources (e.g., Agilent) cannot be used, as the nitrocellulose coating employed with antibody arrays will interfere with the instrument's signal detection system.
- Instrument compatibility:
 - Standard microscope glass slide (25 mm x 75.6 mm x 1 mm)
 - Light filter system reading within the near-red spectrum of Cyanine dyes: Cy3 = excitation 550 nm, emission 570 nm; Cy5 = excitation 659 nm, emission 670 nm

Instrument	Software
Perkin Elmer Packard ScanArray Lite Packard ScanArray Express Packard ScanArray Express HT ProScanArray Microarray Scanner ProScanArray HT Microarray Scanner	ScanArray Express (Packard/Perkin Elmer) ProScanArray Express (Perkin Elmer)
Axon Axon 4200 AL Microarray Scanner	GenePix Pro 6 (Molecular Devices) ArrayPro (Media Cybernetics)
	<i>Other universal software available</i>

Typical scanner settings

- The following parameters are recommended to obtain a signal-noise ratio greater than 10:1.
 - Laser power: 40-80%
 - PMT: 40-80%
- Use the lower settings for the initial scan. Depending on the abundance of protein and therefore signal intensity, the highest settings may accentuate the nitrocellulose background, thereby "washing-out" any positive bound-protein signals.
- Confocal plane focus should be adjusted for arrays from different batches. Nitrocellulose coating thickness may vary between 7-10 μm .

How quickly does the array need to be scanned?

- Cy3/Cy5 is not photo-stable and should be protected from light and scanned within 48 hrs.

What resolution should I scan to obtain a numerical value for each antibody spotted?

- To obtain datapoints of relevance for each antibody, scanner resolution should be set no less than 10 μm .

How do I increase signal intensity?

- Ensure slide is completely dry before scanning. Slides may be air-dried in a positive/laminar flow cabinet. Duration is typically 20 minutes. *NOTE:* do not centrifuge slides, as this may cause the arrayed antibodies to produce a 'comet' effect and subsequently affect data analysis.
- Weak signal intensity may be due to inadequate labeling of the target protein or insufficient protein present in the sample. It is recommended to make certain the D/P ratio is >2 (see Labeling Samples).

- The D/P ratio may be increased by:
 - Increasing the labeling time in half hour increments to a maximum of two hrs
 - Ensuring fresh samples are used
 - Extending the incubation period of the labeled protein on the array for a total of 45 minutes

How do I eliminate high background and improve my signal to noise ratio?

- The background RFU signal will be influenced by the effective dye to protein ratio. Panorama Antibody Arrays are pre-blocked with a proprietary blocker to eliminate background; however, further preventative steps may be required.
- **Using a slide to which a sample has already been applied:**
 - Conduct additional washes of the slide using the wash buffer supplied. This should remove extraneous proteins binding nonspecifically to the nitrocellulose coating.
 - Incubate the slide, covered in foil or in a dark room, for another hour to allow maximum interaction of labeled proteins with the arrayed antibodies.
- **To reduce non-specific binding events:**
 - Add 100 $\mu\text{g}/\text{mL}$ of BSA to either the Washing Buffer or 0.4 M NaCl (final concentration) to reduce non-specific binding events during incubation.
- **Remove excess free dye** – pass labeled sample through Sigma Post-reaction clean-up spin column again.
- **Decrease amount of labeled protein applied to slide** – excessive labeled protein may give a saturated (i.e., white) signal on the array making analysis impossible.

There is a specific antibody of interest that is not generating a signal. What is the problem?

- The abundance of the target protein in the sample may be too low. Presence of the protein and its concentration should be confirmed by Western blotting.

Can I use an alternative method of detection with the antibody array such as HRP-based methods?

It is not recommended to use non-fluorescent based detection methods for the following reasons:

- The arrays were manufactured with BSA labeled with Cy3/Cy5 or monoclonal antibodies to Cy3/Cy5 as positive controls. If alternative methods of detection are used, the controls will not light up and orientation cannot be made, as they are specific for Cy3/Cy5.
- Detection by non-fluorescent methods are typically not sensitive enough to detect low levels of expression change.
- Only one sample can be used per slide because non-fluorescent based detections do not allow for differentiation between a control + sample or multiplexing.

Data Analysis

Are controls spotted on the array?

- Yes, positive and negative controls are spotted in each sub-group on the array.
- Positive control: Monoclonal Anti-Cy3/Cy5 antibodies or BSA labeled with Cy3/Cy5
- Negative control: Unlabeled BSA.
- The .GAL file for antibody position and analysis spreadsheet can be found on the CD provided with the kit or may be downloaded from the Web site: sigma-aldrich.com/arrays.
- Positive controls on the array are for orientation purposes and should not be used for data analysis calculations.

How do I normalize my data?

- Normalization of data may be achieved by determining the variance between the control elements on the array. Antibodies against cytoskeleton protein families (actin, myosins, tubulins) are included on every array as internal controls. Expression of cytoskeleton proteins is normally conserved/unchanged between treated and untreated cell samples.
- The internal control can be the numerical value received from either one or a group of the cytoskeleton antibodies. Results obtained from Cy3/Cy5 labeled samples are then divided by the value determined for the internal control(s). These normalized values can now be used to determine the ratio of up- or down-regulation of particular proteins.
- If one particular family of cytoskeleton proteins exhibits atypical variance, use only those families that show low variance for data normalization.
- Positive control spots are for orientation of the array and should not be used in data analysis calculations.

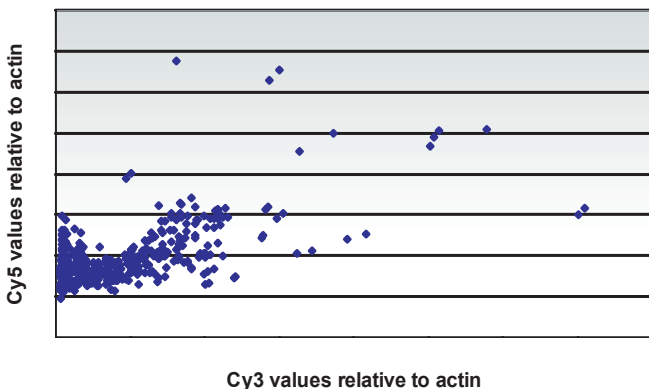


Figure 3. Plot of relative protein expression ratios as normalized against actin controls.

What is the statistically significant difference in fluorescence intensity that indicates a change in protein expression?

- As a general rule, significant change in protein expression is indicated by a two-fold change in fluorescence signal.
- Following normalization, however, some proteins of interest that exhibit slightly less than a two-fold change may warrant further investigation by Western blotting.

Antibody Array Products

Cat. No.	Product Name	Quantity
C5AA1	Panorama Antibody Microarray - Cell Signaling	1 kit
G5AA2	Panorama Antibody Microarray - Gene Regulation	1 kit
M5AA3	Panorama Antibody Microarray - MAPK & PKC Pathways	1 kit
P5AA4	Panorama Antibody Microarray - p53 Pathways	1 kit
XP725	Panorama Antibody Microarray - XPRESS Profiler725	1 kit

Array Specifications

Array slide dimensions:	<ul style="list-style-type: none"> ■ 25 mm x 75.6 mm x 1 mm (standard microscope slide) ■ FAST® slide by Whatman
Diameter of each antibody spot:	Cell Signaling, Gene Regulation, MAPK & PKC, p53 Pathway Arrays <ul style="list-style-type: none"> ■ Each spot is ~0.5 mm ■ Distance between spots is 1 mm ■ Distance between sub-grids is ~1.4 mm XPRESS Profiler725 Array <ul style="list-style-type: none"> ■ Each spot is 0.3 mm ■ Distance between spots is 0.5 mm ■ Distance between sub-grids is ~1.4 mm
Nitrocellulose coating thickness:	<ul style="list-style-type: none"> ■ Typical thickness is 9 µm ■ Batch variance is between 7-10 µm

Publications Referencing Panorama® Antibody Arrays

Links to abstracts can be downloaded at: sigma-aldrich.com/arrayliterature

Armstrong, L. *et al.* (2006) "The role of PI3K/AKT, MAPK/ERK and NF signaling in the maintenance of human embryonic stem cell pluripotency and viability highlighted by transcriptional profiling and functional analysis." *Human Molecular Genetics*, Vol. 15, No. 11, 1894-1913.

Celis, J. *et al.* (2005) "Towards discovery-driven translational research in breast cancer." *FEBS Journal*, Vol. 272, 2-15.

Celis, J. *et al.* (2005) "Identification of Extracellular and Intracellular Signaling Components of the Mammary Adipose Tissue and Its Interstitial Fluid in High Risk Breast Cancer Patients." *Molecular & Cellular Proteomics*, Vol. 4, 492-522.

Kopf, E., and Zharhary, D. (2007) "Antibody Arrays: An emerging tool in cancer proteomics." *Int J Biochem Cell Biol.*, Vol. 39 (7-8), 1305-17.

Kopf, E. *et al.* (2005) "Panorama Ab Microarray Cell Signaling Kit: A unique tool for protein expression analysis." *Proteomics*, Vol. 5, Issue 9, 2412-2416.

Madoz-Gurpide, J., *et al.* (2007) "A proteomics analysis of cell signaling alterations in colorectal cancer." *Molecular and Cellular Proteomics*, Vol. 6, 2150-2164.

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